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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/53, 1/15, A61K 7/13, 7/06, D21C 5/00 // (C12N 1/15, C12R 1:66)	A1	(11) International Publication Number: WO 95/33836 (43) International Publication Date: 14 December 1995 (14.12.95)
(21) International Application Number: PCT/US95/06815		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).
(22) International Filing Date: 31 May 1995 (31.05.95)		
(30) Priority Data: 08/253,781 3 June 1994 (03.06.94) US 08/441,146 15 May 1995 (15.05.95) US		
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(54) Title: PHOSPHONYLDIPEPTIDES USEFUL IN THE TREATMENT OF CARDIOVASCULAR DISEASES

(57) Abstract

The present invention relates to isolated nucleic acid constructs containing a sequence encoding a *Myceliophthora* laccase, and the laccase proteins encoded thereby.

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Phosphonyldipeptides useful in the treatment of cardiovascular diseases

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Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a thermophilic ascomycete, *Myceliophthora*.

15 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper-containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, and perfect forms of *Rhizoctonia*. Laccase exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have

found many potential industrial applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water
5 treatment.

Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these
10 fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi et al. (*Mol. Plant-Microbe Interactions* 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus,
15 *Cryphonectria parasitica*. Kojima et al. (*J. Biol. Chem.* 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (*Experientia* 41: 801, 1985; *PNAS USA* 83: 8854-8858, 1986)
20 have reported the cloning and partial sequencing of the *Neurospora crassa* laccase gene. Saloheimo et al. (*J. Gen. Microbiol.* 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.
25 Attempts to express laccase genes in heterologous fungal systems frequently give very low yields (Kojima et al., *supra*; Saloheimo et al., *Bio/Technol.* 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per
30 liter of active enzyme (Saloheimo, 1991, *supra*). Although laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. At the present time there are no laccases which are expressed at high levels in commercially

utilized hosts such as *Aspergillus*. Thus, the need exists for a laccase which can be produced in commercially useful (i.e., gram per liter or more) quantities. The present invention fulfills such a need.

5

Summary of the Invention

The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Myceliophthora* laccase. The invention also relates to an isolated laccase 10 encoded by the nucleic acid sequence. Preferably, the laccase is substantially pure. By "substantially pure" is meant a laccase which is essentially (i.e., ≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, 15 the invention also provides vectors and host cells comprising the claimed nucleic acid sequence, which vectors and host cells are useful in recombinant production of the laccase. The sequence is operably linked to transcription and translation signals capable of directing expression of 20 the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the construct of the invention, or 25 progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of 30 phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and phenol resin production.

Brief Description of the Figures

Figure 1 shows a restriction map of a 7.5 EcoRI fragment in pRaMB1. The region hybridizing to the *N. crassa* laccase gene probe is shaded.

Figure 2 illustrates the nucleotide (SEQ ID NO: 1) and 5 amino acid (SEQ ID NO: 2) sequence of *Myceliophthora thermophila* laccase. Lower case letters in the nucleotide sequence indicate the position of introns. Putative TATA and CAAT sequences in the promoter region are in boldface and underlined. Consensus lariat structures (PuCTPuAC) within 10 the introns are underlined.

Figure 3 illustrates the construction of plasmid pRaMB5.

Detailed Description of the Invention

15 *Myceliophthora thermophila* is a thermophilic Ascomycete originally described by Apinis (Nova Hedwigia 5: 57-78, 1963) and named *Sporotrichum thermophile*. Subsequent taxonomic revisions have placed this organism in the genus *Chrysosporium* (Von Klopotek, A. Arch. Microbiol. 98: 365-20 369, 1974) and later to *Myceliophthora* (Van Oorschot, Persoonia 9: 401-408, 1977). A number of organisms known by other names also appear to belong to this species. These include *Sporotrichum cellulophilum* (U.S. Patent No. 4,106,989); *Thielavia thermophila* (Fergus and Sinden, Can. 25 J. Botany 47: 1635-1637, 1968); *Chrysosporium fergusii* and *Corynascus thermophilus* (Von Klopotek, *supra*). This species is known as a source of a number of different industrially useful enzymes, such as cellulases, β -glucosidase and xylanase (see, e.g., Oberson et al., Enzyme 30 Microb. Technol. 14 : 303-312, 1992; Merchant et al., Biotechnol. Lett. 10: 513-516, 1988; Breuil et al., Biotechnol. Lett. 8: 673-676, 1986; Gilbert et al., Bioresource Technol. 39: 147-154, 1992). It has now been determined that *Myceliophthora* produces a neutral pH

laccase, and the gene encoding this laccase can be used to produce large yields of the enzyme in convenient host systems such as *Aspergillus*.

To identify the presence of a laccase gene in

5 *Myceliophthora*, a 5' portion of the *Neurospora crassa* laccase gene (*lcc1*) is used as a probe, under conditions of mild stringency, in southern hybridization of total genomic DNA of different fungal species. An approximately 12 kb laccase specific sequence is detected in the *Myceliophthora*

10 DNA. The *N. crassa* fragment is then used to screen about 20,000 plaques of an *M. thermophila* genomic DNA library in a λ EMBL4 bacteriophage cloning vector. Eight plaques strongly hybridize with the probe; from these eight, DNA is isolated from three. Each of these clones contains a 7.5 EcoRI

15 fragment which also hybridizes to the probe (Figure 1). One of the fragments is subcloned into pBR322 to generate plasmid pRaMB1. Using the *lcc1* probe, the position of the coding region of the clone is determined. The entire *M. thermophila* coding region appears to be contained within a 3.2

20 kb *NheI-BglII* segment, which is then cloned into pUC119 and sequenced by the primer walking method.

Once the sequence is determined, the positions of introns and exons within the gene is assigned based on alignment of the deduced amino acid sequence to the

25 corresponding *N. crassa* laccase gene product. From this comparison, it appears that the gene (*lccM*) of *M. thermophila* is composed of seven exons (246, 79, 12, 70, 973, 69 and 411 nucleotides) interrupted by six introns (85, 84, 102, 72, 147, and 93 nucleotides). The coding region,

30 excluding intervening sequences, is very GC-rich (65.5% G+C) and encodes a preproenzyme of 620 amino acids: a 22 amino acid signal peptide, a 25 amino acid propeptide, and a mature laccase comprising 573 amino acids. The sequence of

the *M. thermophila* gene and the predicted amino acid sequence is shown in Figure 2 (SEQ ID NOS: 1 and 2).

The laccase gene is then used to create an expression vector for transformation of *Aspergillus* host cells. The 5 vector, pRaMB5 contains the *A. oryzae* TAKA-amylase promoter and terminator regions. The construction of pRaMB5 is outlined in Figure 3. *Aspergillus* cells are cotransformed with the expression vector and a plasmid containing the *pyrG* or *amds* selectable marker. Transformants are selected on 10 the appropriate selective medium containing ABTS. Laccase-producing colonies exhibit a green halo and are readily isolatable. Selected transformants are grown up in shake flasks and culture broths tested for laccase activity by the syringaldazine method. Shake flask cultures are capable of 15 producing 0.2 or more g/liter of laccase, and in fermentors, yields of over 1-2 g/liter are observed.

According to the invention, a *Myceliophthora* gene encoding a laccase can be obtained by methods described above, or any alternative methods known in the art, using 20 the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell 25 independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, 30 optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be used

according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene,

5 include but are not limited to the prokaryotic β -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from
10 recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of
15 vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, or
20 an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

25 In the vector, the laccase DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the
30 host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the lac operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*

α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB*

5 genes. In a yeast host, a useful promoter is the *eno-1* promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase,

10 *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and *glaA* promoters.

15 The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention.

Termination and polyadenylation sequences may suitably be
20 derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

25 The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such
30 as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amds*, *pyrG*, *argB*, *niaD*, *SC*, and *hygB*, a marker giving rise to hygromycin resistance. Preferred for use in

an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as
5 described in WO 91/17243.

It is generally preferred that the expression gives rise to a product which is extracellular. The laccases of the present invention may thus comprise a preregion
10 permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective
15 preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the α -factor from *Saccharomyces cerevisiae* or the calf
20 preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the maltogenic amylase from *Bacillus NCIB 11837*, *B. stearothermophilus* α -amylase, or *Bacillus licheniformis* subtilisin. An effective signal
25 sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements,
30 respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al.. Molecular Cloning, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the 5 recombinant production of an enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more 10 likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in 15 connection with the different types of host cells.

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus laetus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in 25 a manner known *per se*.

The host cell may also be a eukaryote, such as 30 mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces*

cerevisiae. Useful filamentous fungi may selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell.

5 Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of
10 transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above
15 under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the
20 invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

In a preferred embodiment, the recombinant production
25 of laccase in culture is achieved in the presence of an excess amount of copper. Although trace metals added to the culture medium typically contain a small amount of copper, experiments conducted in connection with the present invention show that addition of a copper supplement to the
30 medium can increase the yield of active enzyme many-fold. Preferably, the copper is added to the medium in soluble form, preferably in the form of a soluble copper salt, such as copper chloride, copper sulfate, or copper acetate. The final concentration of copper in the medium should be in the

range of from 0.2-2mM, and preferably in the range of from 0.05-0.5mM. This method can be used in enhancing the yield of any recombinantly produced fungal laccase, as well as other copper-containing enzymes, in particular
5 oxidoreductases.

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA α -amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474, 1984).

Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figure 1. It will also be apparent that the invention encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figure 1, but which differ from the specifically depicted nucleotide sequences

by virtue of the degeneracy of the genetic code. Also, reference to Figure 1 in the specification and the claims will be understood to encompass both the genomic sequence depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be understood to mean a DNA molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

The *Myceliophthora* laccase described herein has a particularly high specific activity on a syringaldazine substrate relative to other known ascomycete or deuteromycete extracellular laccases in which such specific activity has been described. The present sequence provides a means by which other such ascomycete and/or deuteromycete laccases can also be isolated. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available ascomycete and deuteromycete strains. In particular, the specific sequence disclosed herein can be used to design primers and/or probes useful in isolating similar laccase genes by standard PCR or southern hybridization techniques. The present invention thus encompasses those ascomycete and deuteromycete laccases which have a specific activity of at least about 30 SOU/mg, and preferably at least about 40 SOU/mg, "SOU" being defined as μ mole of substrate oxidized per minute as measured with syringaldazine as a substrate, at optimum pH.

In addition, the invention also encompasses other *Myceliophthora* laccases, including alternate forms of

laccase which may be found in *M. thermophila* and as well as laccases which may be found in other fungi falling within the definition of *Myceliophthora* as defined by Van Oorschot, 1977, *supra*. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available *Myceliophthora* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in isolating laccase genes by standard PCR or southern hybridization techniques. Other named *Myceliophthora* species include *Myceliphthora hinnulea* (Awao et al., *Mycotaxon*. 16: 436-440, 1983), *Myceliophthora vellerea* (Guarro et al., *Mycotaxon*. 23: 419-427, 1985), and *Myceliophthora lutea* Costatin. Also encompassed are laccases which are synonyms, e.g., anamorphs or perfect states of species or strains of the genus *Myceliophthora*. Strains of *Myceliophthora* are readily accessible to the public in a number of culture collections, such as ATCC 48102, 48103, 48104 et al.; CBS 117.65, 131.65, 379.65 et al., DSM 1799 (*M. thermophila*), ATCC 52474, CBS 539.82, 540.82 et al. (*M. hinnulea*), DSM 62114, CBS 146.50, 147.50, 157.51 et al (*M. lutea*), and CBS 478.76, 479.76 and 715.84 (*M. vellerea*). The invention also encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80%, preferably at least 85%, and most preferably at least 90-95% homology with the amino acid sequence depicted in Figure 1, and which qualitatively retains the laccase activity of the sequence described herein. Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted

by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are 5 conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be 10 determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in 15 order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., Holzforschung 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; 20 PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of 25 chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 30 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976. Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406; WO 92/18683; EP 0495836; Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversiteit Gent. 56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem. 42: 273-282, 1991.

The laccase is particularly well-suited for use in hair dyeing. In such an application, the laccase is contacted with a dye precursor, preferably on the hair, whereby a controlled oxidation of the dye precursor is achieved to convert the precursor to a dye, or pigment producing compound, such as a quinoid compound. The dye precursor is preferably an aromatic compound belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphthols) and the phenols. The dye precursors can be used alone or in combination. At least one of the intermediates in the copolymerization must be an ortho- or para-diamine or aminophenol (primary intermediate). Examples of such are found in Section IV, below, and include p-phenylene-diamine (pPD), p-toluylene-diamine, chloro-p-phenylenediamine, p-aminophenol, o-aminophenol. 3,4-diaminotoluene; additional compounds are also described in US Patent No. 3,251,742, the contents of which are incorporated herein by reference. In one embodiment, the starting materials include not only the enzyme and a primary intermediate, but also a modifier (coupler) (or combination of modifiers), which modifier is typically a meta-diamine, meta-aminophenol, or a polyphenol. Examples of modifier

compounds include m-phenylene-diamine, 2,4-diaminoanisole, α-naphthol, hydroquinone, pyrocatechol, resorcinol, and 4-chlororesorcinol. The modifier then reacts with the primary intermediate in the presence of the laccase,

5 converting it to a colored compound. In another embodiment, the laccase can be used with the primary intermediate directly, to oxidize it into a colored compound. In all cases, the dyeing process can be conducted with one or more primary intermediates, either alone or in combination with

10 one or more modifiers. Amounts of components are in accordance with usual commercial amounts for similar components, and proportions of components may be varied accordingly.

The use of this laccase is an improvement over the more traditional use of H₂O₂, in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with laccase can be conducted at alkaline, neutral or even acidic pH, and the oxygen needed for oxidation comes from the air,

20 rather than via harsh chemical oxidation. The result provided by the use of the *Myceliophthora* laccase is comparable to that achieved with use of H₂O₂, not only in color development, but also in wash stability and light fastness. An additional commercial advantage is that a

25 single container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of H₂O₂.

The present laccase can also be used for the polymerization of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as

30 apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such

applications have been described in Stutz, *Fruit processing* 7/93, 248-252, 1993; Maier et al., *Dt. Lebensmittelrindschau* 86(5): 137-142, 1990; Dietrich et al., *Fluss. Obst* 57(2): 67-73, 1990..

5 Laccases such as the *Myceliophthora laccase* are also useful in soil detoxification (Nannipieri et al., *J. Environ. Qual.* 20: 510-517, 1991; Dec and Bollag, *Arch. Environ. Contam. Toxicol.* 19: 543-550, 1990).

10 The invention is further illustrated by the following non-limiting examples.

EXAMPLES

I. ISOLATION OF MYCELIOPHTHORA THERMOPHILA LACCASE GENE

15 A. MATERIALS AND METHODS

1. DNA Extraction and Hybridization analysis

Total cellular DNA is extracted from fungal cells of *Myceliophthora thermophila* strain E421 grown 24 hours in 25 ml of YEG medium (0.5% yeast extract, 2% glucose) using the 20 following protocol: mycelia are collected by filtration through Miracloth (Calbiochem) and washed once with 25 ml of TE buffer. Excess buffer is drained from the mycelia which are subsequently frozen in liquid nitrogen. Frozen mycelia are ground to a fine powder in an electric coffee grinder, and the powder added to 20 ml of TE buffer and 5 ml of 20% SDS (w/v) in a disposable plastic centrifuge tube. The mixture is gently inverted several times to ensure mixing, and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Sodium acetate 30 (3M solution) is added to give a final concentration of 0.3 M and the nucleic acids are precipitated with 2.5 volumes of ice cold ethanol. The tubes are centrifuged at 15,000 x g for 30 minutes and the pellet is allowed to air-dry for 30 minutes before resuspending in 0.5 ml of TE buffer. DNase-

free ribonuclease A is added to a concentration of 100 μ g/ml and the mixture is incubated at 37°C for 30 minutes. Proteinase K (200 μ g/ml) is added and each tube is incubated an additional one hour at 37°C. Finally, each sample is
5 extracted twice with phenol:chloroform:isoamyl alcohol before precipitating the DNA with sodium acetate and ethanol. DNA pellets are dried under vacuum, resuspended in TE buffer, and stored at 4°C.

Total cellular DNA samples from transformants and an
10 untransformed control strain are analyzed by Southern hybridization. Approximately 5 μ g of DNA is digested with EcoRI and fractionated by size on a 1% agarose gel. The gel is photographed under short wavelength UV and soaked for 15 minutes in 0.5 M NaOH, 1.5 M NaCl followed by 15 minutes in
15 1 M Tris-HCl, pH 8, 1.5 M NaCl. DNA in the gel is transferred onto Zeta-Probe™ hybridization membrane (BioRad Laboratories) by capillary blotting in 20 X SSPE (R. W. Davis et al., Advanced Bacterial Genetics, A Manual for Genetic Engineering. Cold Spring Harbor Press. 1980)
20 Membranes are baked for 2 hours at 80°C under vacuum and soaked for 2 hours in the following hybridization buffer at 45°C with gentle agitation: 5X SSPE, 35% formamide (v/v), 0.3% SCS, 200 μ g/ml denatured and sheared salmon testes DNA. The laccase-specific probe fragment (approx. 1.5 kb)
25 encoding the 5'-portion of the *N. crassa* *lcc1* gene is amplified from *N. crassa* genomic DNA using standard PCR conditions (Perkin-Elmer Cetus, Emeryville, CA) with the following pair of primers: forward primer, 5'
CGAGACTGATAACTGGCTTGG 3'; reverse primer, 5'
30 ACGGCGCATTCAGGGAAAGT 3'. The amplified DNA segment is first cloned into a TA-cloning vector (Invitrogen, Inc., San Diego, CA), then purified by agarose gel electrophoresis following digestion with EcoRI. The purified probe fragment is radiolabeled by nick translation with α [³²P]dCTP (Amersham)

and added to the hybridization buffer at an activity of approximately 1×10^6 cpm per ml of buffer. The mixture is incubated overnight at 45°C in a shaking water bath.

Following incubation, the membranes are washed once in 0.2 x 5 SSPE with 0.1% SDS at 45°C followed by two washes in 0.2 x SSPE (no SDS) at the same temperature. The membranes are allowed to dry on paper towels for 15 minutes, then wrapped in Saran Wrap™ and exposed to x-ray film overnight at -70°C with intensifying screens (Kodak).

10 2. DNA Libraries and Identification of Laccase Clones

Genomic DNA libraries are constructed in the bacteriophage cloning vector λ -EMBL4 (J.A. Sorge, in Vectors, A Survey of Molecular Cloning Vectors and Their Uses, Rodriguez et al., eds, pp. 43-60, Butterworths, Boston, 15 1988). Briefly, total cellular DNA is partially digested with Sau3A and size-fractionated on low-melting point agarose gels. DNA fragments migrating between 9kb and 23 kb are excised and eluted from the gel using β -agarase (New England Biolabs, Beverly MA). The eluted DNA fragments are 20 ligated with BamHI-cleaved and dephosphorylated λ -EMBL4 vector arms, and the ligation mixtures are packaged using commercial packaging extracts (Stratagene, LaJolla, CA). The packaged DNA libraries are plated and amplified on *Escherichia coli* K802 cells. Approximately 10,000-20,000 25 plaques from each library are screened by plaque-hybridization with the radiolabeled *lcc1* DNA fragment using the conditions described above. Plaques which give hybridization signals with the probe are purified twice on *E. coli* K802 cells, and DNA from the corresponding phage is 30 purified from high titer lysates using a Qiagen Lambda kit (Qiagen, Inc., Chatsworth, CA).

3. Analysis of Laccase Genes

Restriction mapping of laccase clones is done using standard methods (Lewin, *Genes*. 2d ed., Wiley & Sons, 1985, New York). DNA sequencing is done with an Applied Biosystems Model 373A automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using the primer walking technique with dye-terminator chemistry (H. Giesecke et al., *J. Virol. Methods* 38: 47-60, 1992). Oligonucleotide sequencing primers are synthesized on an Applied Biosystems model 394 DNA/RNA Synthesizer.

10

B. RESULTS AND DISCUSSION

1. Identification of Laccase Gene Sequence

Total cellular DNA samples are prepared from the species *Neurospora crassa*, *Botrytis cinerea*, and *Myceliophthora*. Aliquots of these DNA preparations are digested with *Bam*HI and fractionated by agarose gel electrophoresis. DNA in the gel is blotted to a Zeta-Probe™ membrane filter (BioRad Laboratories, Hercules, CA) and probed under conditions of mild stringency with a radiolabeled fragment encoding a portion of the *N. crassa* *lcc1* gene, as described above. Laccase-specific sequences are detected in the genomes of *M. thermophila* and the *N. crassa* control, but not in the *B. cinerea* genomic DNA with this probe.

25

2. Cloning and Characterization of Myceliophthora thermophila Laccase (MtL) Gene

Approximately 20,000 plaques from a *M. thermophila* genomic DNA library constructed in a λ -EMBL4 cloning vector are screened. The library is composed of approximately 30 10,000 independent clones with inserts ranging in size from 9kb to 23kb. Assuming an average insert size of 10 kb and a total genome size of 4×10^7 bp for *M. thermophila*, this figure is about 2.5 times the number of clones required to represent the entire genome. Eight plaques are identified

that hybridized strongly to the *N. crassa* laccase gene probe. DNA is isolated from three of these, cleaved with EcoRI and analyzed by agarose gel electrophoresis and Southern hybridization. All three of these clones contain a 5 7.5 kb EcoRI fragment which hybridized to the laccase-specific probe. One of these EcoRI fragments is subcloned into pBR322 (Bolivar et al., Gene 2: 95-113, 1977) to generate plasmid pRaMB1. A restriction map of this DNA segment is shown in Fig. 1. The position of the laccase 10 coding region on this clone is determined by hybridization with the lcc1 gene fragment described above. Based on mapping data obtained, and an estimated size of the laccase protein of approximately 80 kdal, it is reasoned that the entire *M. thermophila* laccase coding region is contained 15 with a 3.2 kb NheI-BglII segment which is then subcloned into pUC119 (Viera and Messing, Methods Enzymol. 153: 3-11, 1987). The nucleotide sequence of this segment is determined using the primer walking method (Giesecke et al., supra). The nucleic acid sequence is shown in Figure 2 and 20 SEQ ID NO: 1.

The deduced amino acid sequence of MtL is obtained on the basis of amino acid sequence homology with the *N. crassa* laccase. At the amino acid level, these two laccases share approximately 60% sequence identity. Similarity is highest 25 in regions that correspond to the four histidines and one cysteine which are involved in the formation of the trinuclear copper cluster (Perry et al., J. Gen. Microbiol. 139: 1209-1218, 1993; Coll et al. Appl. Environ. Microbiol. 59: 4129-4135, 1993; Messerschmidt et al. J. Mol. Biol. 206: 30 513-530, 1989). There are 11 potential sites for N-linked glycosylation in the deduced amino acid sequence of MtL. the first 22 amino acids of MtL appear to comprise a canonical signal peptide with a predicted cleavage following an Ala residue (vonHeijne, J. Mol. Biol. 173:243-251, 1984).

Although the amino terminal sequence of the native MtL is unknown, the amino terminus of recombinant MtL produced in *A. oryzae* is blocked with a pyro-glutamate residue. Enzymatic removal of this residue followed by amino acid sequencing suggests that mature MtL begins with a Gln residue (position 1 in Figure 2; SEQ ID NO: 2). Thus, MtL is apparently synthesized as a 620 amino acid preproenzyme having a 22 amino acid signal peptide and propeptide of 25 residues. *Neurospora crassa* laccase(NcL) is processed similarly at its amino terminal end. In addition, NcL is also proteolytically processed at its C-terminus, resulting in the removal of 13 amino acids (Germann et al. J. Biol. Chem. 263: 885-896, 1988). The processing site is contained within the sequence Asp-Ser-Gly-Leu*Arg₅₅₈ (where * designates the cleavage site). A similar sequence exists near the C-terminal end of MtL(Asp-Ser-Gly-Leu-Lys₅₆₀), suggesting the *Myceliophthora* enzyme may also be subject to C-terminal processing (Asp-Ser-Gly-Leu*lys₅₆₀) which would remove 12 amino acids.

The positions of six introns (85, 84, 102, 72, 147, and 93 nucleotides) within the *lcc1* coding region are determined by comparing the deduced amino acid sequence of MtL to that of NcL and by applying the consensus rules for intron features in filamentous fungi (Gurr et al., in Gene Structure in Eukaryotic Microbes, J.R. Kinghorn, ed.) pp 93-139, IRL Press, Oxford, 1987). The 1860 nucleotides of coding sequence, excluding introns, are rich in guanosine and cytosine (65.5% G+C). The codon usage pattern for this gene reflects the DNA base composition in a strong bias(89.7%) for codons ending in G or C.

III. EXPRESSION OF MYCELIOPHTHORA LACCASE IN ASPERGILLUS

A. MATERIALS AND METHODS

1. Bacterial and Fungal Host Strains

Escherichia coli JM101 (Messing et al., Nucl. Acids Res. 9:309-321, 1981) is used as a host for construction and routine propagation of laccase expression vectors in this study. Fungal hosts for laccase expression included the 5 *Aspergillus niger* strains Bo-1, AB4.1 and AB1.13 (Mattern et al., Mol. Gen. Genet. 234: 332-336), as well as a uridine-requiring (*pyrG*) mutant of the α -amylase-deficient *Aspergillus oryzae* strain HowB104.

2. Plasmids

10 Plasmid pRaMB2 is a pUC119 derivative which contains a 3.2 kb *Bgl*III-*Nhe*I fragment of *M. thermophila* genomic DNA encoding MtL. The vector pMWR is constructed by inserting the *A. oryzae* TAKA-amylase promoter and terminator elements from pTAKA17 (Christensen et al., Bio/Technol. 6: 1419-1422, 15 1988; EP 238 023) into pUC18 (Yanisch-Perron et al., Gene 33: 103-119, 1985). In this vector, there is a unique *Swa*I site at the end of the promoter element and a single *Nsi*I site at the beginning of the terminator for directional cloning of coding sequences. The cloning vehicle pUC518 is derived by 20 inserting a small linker containing *Nsi*I, *Cla*I, *Xho*I, and *Bgl*III restriction sites between the adjacent *Bam*HI and *Xba*I sites of pUC118 (Vieira and Messing, *supra*). Plasmid pToC68 (WO 91/17243) contains the *A. oryzae* TAKA-amylase promoter and *A. niger* *glaA* terminator, and pToC90 (WO 25 91/17243) carries the *A. nidulans* *amdS* gene.

3. Construction of Laccase Expression Vectors

The construction strategy for the laccase expression vector pRaMB5 is outlined in Figure 3. The promoter directing transcription of the laccase gene is obtained from 30 the *A. oryzae* α -amylase (TAKA-amylase) gene (Christensen et al., *supra*), as well as the TAKA-amylase terminator region. The plasmid is constructed first by modifying pMWR3 by inserting a small linker which contains an *Apal* site between

the *SwaI* and *NsiI* sites, creating a plasmid called pMWR3-SAN. *PfuI* polymerase-directed PCR (Stratagene, La Jolla, CA) is used to amplify a short DNA segment encoding the 5'-portion of MtL, from the start codon to an internal *PstI* site (approximately 0.5 kb). The forward primer for this PCR reaction is designed to create an *EcoRI* site just upstream of the start codon. Next, the amplified fragment is digested with *EcoRI* and *PstI* [during this step, the *EcoRI* site is made blunt by treatment with dNTPs and DNA 5 polymerase I (Klenow fragment)] and purified by agarose gel electrophoresis. The 3' portion of the *M. thermophila* coding region is excised from pRaMB2 as a 2kb *PstI-ApaI* fragment (this segment also contains approximately 110 bp from the 3'-untranslated region). These two fragments are. 10 combined with *SwaI*- and *ApaI*-cleaved pMWR3-SAN in a three-part ligation reaction to generate the laccase expression vector pRaMB5.

4. Transformation of *Aspergillus* host cells

Methods for co-transformation of *Aspergillus* strains 20 are as described in Christensen et al., *supra*. For introduction of the laccase expression vectors into *A. oryzae* HowB 104 *pyrG*, equal amounts (approximately 5 µg each) of laccase expression vector and one of the following plasmids 25 are used: pPYRG (Fungal Genetics Stock Center, Kansas City, KS) which contains the *A. nidulans* *pyrG* gene (Oakley et al., Gene 61385-399, 1987); pSO2 which harbors the clones *A. oryzae* *pyrG* gene; pPRYG24 which contains the *A. ficuum*(=*A. niger*) *pyrG* gene. Protrophic (Pyr+) transformants are selected 30 on *Aspergillus* minimal medium (Rowlands and Turner, Mol. Gen. Genet. 126: 201-216, 1973), and the transformants are transformants are screened for the ability to produce laccase on minimal medium containing 1 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) [ABTS]. Cells which

secrete active laccase oxidize the ABTS, producing a green halo surrounding the colony. Lastly, *A. niger* Bo-1 protoplasts are co-transformed using equal amounts (approximately 5 μ g each) of laccase expression vector and 5 pToC90 which contains the *A. nidulans* *amds* (acetamidase) gene (Hynes et al., Mol. Cell Biol. 3: 1430-1439, 1983). AmdS⁺ transformants are selected on Cove minimal medium (Cove, Biochim. Biophys. Acta 113: 51-56, 1966) with 1% glucose as the carbon source and acetamide as the sole 10 nitrogen source and screened for laccase expression on cove medium with 1 mM ABTS.

5. Analysis of Laccase-Producing Transformants

Transformants which produce laccase activity on agar plates are purified twice through conidiospores and spore 15 suspensions in sterile 0.01% Tween-80 are made from each. The density of spores in each suspension is estimated spectrophotometrically (A_{595} nm). Approximately 0.5 absorbance units of spores are used to inoculate 25 ml of ASPO4 or MY50 medium in 125 ml plastic flasks. The cultures 20 are incubated at 37°C with vigorous aeration (approximately 200 rpm) for four to five days. Culture broths are harvested by centrifugation and the amount of laccase activity in the supernatant is determined using syringaldazine as a substrate. Briefly, 800 μ l of assay 25 buffer (25 mM sodium acetate, pH 5.5, 40 μ M CuSO₄) is mixed with 20 μ l of culture supernatant and 60 μ l of 0.28 mM syringaldazine (Sigma Chemical Co., St. Louis, MO) in 50% ETOH. The absorbance at 530 nm is measured over time in a Genesys 5 UV-vis spectrophotometer (Milton-Roy). One 30 laccase unit (LACU) is defined as the amount of enzyme which oxidizes one μ mole of substrate per minute at room temperature. SDS-polyacrylamide gel electrophoresis (PAGE) is done using precast 10-27% gradient gels from Novex (San

Diego, CA). Protein bands are developed using Coomassie Brilliant Blue(Sigma).

B.RESULTS AND DISCUSSION

5 1. Expression of *Myceliophthora laccase*

Laccase-producing transformants are detected by incorporation of ABTS into selective media. Using *pyrG* or *amds* as the selectable marker, co-transformation frequencies vary from about 30% to 70%. Heterologous expression of MtL 10 appears to be highest in *A. oryzae* transformants. Furthermore, production appears to be better in ASPO4 medium compared to MY50, although the reasons for this are unknown. SDS-PAGE analysis of culture broth samples shows a prominent laccase band at approximately 80 kdal, which is similar to. 15 the size of the native enzyme purified from *M. thermophila*. Similar analysis of the culture filtrates from *A. niger* Bo-transformants indicate that the laccase band is obscured by very intense glucoamylase and acid-stable amylase protein bands. Results are shown in Table 1.

Table 1. MTL expression among selected *A. oryzae* and *A. niger* transformants

	HOST STRAIN	TRANSFORMANT	TRANSFORMING DNAs	MTLACU/ML	
				ASPO4	MY50
5	<i>A. oryzae</i> HowB104 <i>pyrG</i>	untransformed	none	0.00	0.00
		RaMB5.15	pRaMB5+pPYRG	0.85	0.29
		RaMB5.30	pRaMB5+pPYRG	0.71	0.87
		RaMB5.33	pRaMB5+pPYRG	0.60	0.26
		RaMB5.108	pRaMB5+PSO2	0.68	0.19
		RaMB5.111	pRaMB5+PSO2	0.70	0.17
		RaMB5.121	pRaMB5+PSO2	0.49	0.20
		RaMB5.142	pRaMB5+PSO2	0.54	0.04
	<i>A. Niger</i> Bo-1	untransformed	none	0.00	0.00
		RaMB5.1	pRaMB5+pToC90	n.d.	0.20
		RaMB5.25	pRaMB5+pToC90	n.d.	0.09
		RaMB5.49	pRaMB5+pToC90	n.d.	0.06
		RaMB5.51	pRaMB5+pToC90	n.d.	0.12
		RaMB5.53	pRaMB5+pToC90	n.d.	0.21
		RaMB5.62	pRaMB5+pToC90	n.d.	0.16

n.d.= not determined

2. Expression in the presence or absence of excess copper

A 1 ml aliquot of a spore suspension of *Aspergillus oryzae* transformant HowB104-pRaMB5.30 (approximately 10^9 spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (maltose, 50g/l; $MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4 , 10g/l; K_2SO_4 , 2g/l; $CaCl_2 \cdot 2H_2O$ 0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; trace metals [$ZnSO_4 \cdot 7H_2O$, 14.3 g/l; $CuSO_4 \cdot 5H_2O$, 2.5 g/l; $NiCl_2 \cdot 6H_2O$, 10 0.5 g/l; $FeSO_4 \cdot 7H_2O$, 13.8 g/l, $MnSO_4 \cdot H_2O$, 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18 hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media ($MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4 , 2g/l; citric acid 4g/l; K_2SO_4 , 3g/l; $CaCl_2 \cdot 2H_2O$, 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitiation rate is maintained between 600 and 1300 rpm at approximately the minimum level required to maintain the dissolved oxygen level in the culture above 20%. Sterile feed (Nutriose 725[maltose syrup], 225 g/l; urea, 30 g/l; yeast extract, 15 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 30 g of feed 30 is added initially before inoculation; 0-24 h, 2 g/l h; 24-48 h, 4 g/l h, 48h-end, 6 g/l.

Copper is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank

to a final level of 0.5 mM. The fermentation described above is also conducted without the addition of copper supplement to the tank medium. Samples for enzyme activity determination are withdrawn and filtered through Miracloth 5 to remove mycelia. These samples are assayed for laccase activity by the LACU assay described above. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 45 LACU/ml achieved after 180 hours in the fermentation 10 containing excess copper. At a specific activity of 22 LACU/mg, this corresponds to 2g/l of recombinant laccase expressed. On the other hand, the maximum laccase activity achieved in the fermentation without copper supplement is approximately 10 LACU/ml after 170 hours, or about 25% of 15 that found in the presence of additional copper.

III. PURIFICATION AND CHARACTERIZATION OF MYCELIOPHTHORA

LACCASE

A. MATERIALS AND METHODS

20 1. Materials
Chemicals used as buffers and substrates are commercial products of at least reagent grade. Endo/N-glycosidase F and pyroglutamate amino peptidase are purchased from Boehringer Mannheim. Chromatography is performed on either 25 a Pharmacia FPLC or a conventional low pressure system. Spectroscopic assays are conducted on either a spectrophotometer(Shimadzu PC160) or a microplate reader(Molecular Devices). Britton & Robinson(B&R) buffers are prepared according to the protocol described in Quelle, 30 Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964.

2. Enzymatic Assay

Laccase activity is determined by syringaldazine oxidation at 30°C in a 1-cm quartz cuvette. 60µl

syringaldazine stock solution (0.28 mM in 50% ethanol) and 20 μ l sample are mixed with 0.8 ml preheated buffer solution. The oxidation is monitored at 530nm over 5 minutes. The activity is expressed as μ mole substrate oxidized per minute. B&R buffers with various pHs are used. The activity unit is referred to here as "SOU". A buffer of 25 mM sodium acetate, 40 μ M CuSO₄, pH 5.5, is also used to determine the activity, which is referred to as LACU, as defined above. 2,2'-azinobis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) oxidation assays are done using 0.4 mM ABTS, B&R buffer, pH 4.1, at room temperature by monitoring ΔA_{405} . An ABTS oxidase activity overlay assay is performed by pouring cooled ABTS-agarose(0.05 g ABTS, 1 g agarose, 50 ml H₂O, heated to dissolve agarose) over a native IEF gel and incubating at room temperature. Thermostability analysis of the laccase(r-MtL) is performed using samples that have 3 SOU activity pre-incubated in B&R buffer, pH 6, at various temperatures. Samples are assayed after a 400-fold dilution into the same buffer at room temperature.

20 3. Purification from a fermentor broth

3.7 liters of cheese-cloth filtered broth (pH 7.6, 16 mS) is filtered through Whatman #2 filter paper. The broth is concentrated on a Spiral Concentrator (Amicon) with a SLY100 membrane (MWCO:100) from 3700 ml to 200 ml. The concentrate is adjusted to 0.75 mS by diluting it in water and reconcentrated on SLY100 to 170 ml. The washed and concentrated broth has a dense greenish color.

The broth is frozen overnight at -20°C, thawed the next day and loaded onto a Q-sepharose XK26 column (120 ml), pre-equilibrated with 10 mM Tris, pH 7.5, 0.7 mS(Buffer A). The blue laccase band migrates slowing down the column during loading. One group of blue fractions runs through the column after loading and washing by Buffer A. A second group eluted during the linear gradient with Buffer B

(Buffer A plus 2 M NaCl). Some brown material with no laccase activity is eluted out later with 1 M NaOH. SDS-PAGE analysis shows that this preparation results in pure laccase.

5 4. Analyses of amino acid content, extent of glycosylation, and N-terminal sequence

N-terminal sequencing is performed on an ABI 476A sequencer. Total amino acid analysis, from which the extinction coefficient of r-MtL is determined, is performed 10 on a HP AminoQuant instrument. Deglycosylation is done using endo/N-glucosidase F according to the manufacturer's instructions and carbohydrate content is estimated by mobility difference as determined on SDS-PAGE. N-terminus de-blocking with pyroglutamate amino peptidase is carried 15 out according to manufacturer's instructions. About 80 μ g r-MtL is treated with 4 μ g peptidase with or without the presence of 1 M urea or 0.1 M guanidine HCl before being blotted on a PVDF membrane for sequencing. About 20 pmol de-blocked protein is obtained and sequenced.

20 SDS-PAGE and native IEF analysis are performed on either a Novex cell or a Mini Protean II and a Model 111 Mini IEF cells (Bio-Rad). Gel filtration analyses are done on a Sephadryl S-300 (Pharmacia), from which the native MW is estimated by using Blue Dextran (2000 kdal), bovine IgG (158 kdal), bovine serum albumin (66 kdal), ovalbumin (45 kdal) and horse heart myoglobin(17 kdal) to calibrate the column.

B. RESULTS AND DISCUSSION

1. Purification and characterization of r-MtL from a fermentor broth

30 From 3.7 l of fermentor broth, about 2-3 g of r-MtL are isolated. Initial concentration using a membrane with MWCO of 100 kdal removed significant amounts of brown material and small contaminant proteins. The low affinity of r-MtL toward Q-Sepharose matrix equilibrated with 10 mM Tris, pH

7.5, facilitates its separation from other more acidic and more tightly bound impurities. As shown by SDS-PAGE, this preparation resulted in essentially pure laccase for the most active fractions located around the peak. Other less 5 active fractions can be further purified on either Mono-Q with a shallower gradient or a gel filtration column, such as S-300, from which the contaminants are separated due to their smaller MW. An overall 18-fold purification and a recovery of 67% are achieved. As discussed below, the 10 existence of two elution bands of r-MtL on Q-Sepharose chromatogram is probably due to a differential glycosylation.

The purified r-MtL shows a MW of 100-140 kdal on S-300 gel filtration and a MW of 85 kdal on SDS-PAGE. The 15 increase of r-MtL mobility on SDS-PAGE after deglycosylation suggests that carbohydrates account for 14% of its total mass. Native IEF shows a major band at pI ~4.2 that is active in ABTS overlay assay.

Directly sequencing the N-terminus of the purified r- 20 MtL from samples either in desalted solution or on PVDF membrane are unsuccessful. However, treatment of r-MtL with pyroglutamate amino peptidase yielded a protein with deblocked N-terminus. This suggests the processing of a propeptide during the maturation of r-MtL, a 25 posttranslational event similar to that of *N. crassa* laccase but not found in other laccases such as *Rhizoctonia solani*. The proposed scheme is outlined below.

MKSFISAATLWIVGILTPSVAAPPSTEPQRDLLVPITEREEAAVKARQQSCNTPS
30 |<-putative signal peptide->|<- putative propeptide ->|<-N-terminus

The spectrum of the blue r-MtL has absorption maxima at 276 and 589 nm.

The activity of the laccase is tested by using either syringaldazine and ABTS as substrates. Expressed as per Abs₂₇₆ or per mg, the laccase has a value of 20 or 45 units for SOU at pH 6.5, respectively. The LACU assay yields a 5 value of 10 or 22 units per Abs₂₇₆ or per mg.

The pH profile of r-MtL activity is quite close to that of the wild type, with an optimal pH of 6.5. The upper temperature limit for retaining full activity after a 20 minute preincubation observed for r-MtL is approximately 10 60°C. The purified r-MtL shows no activity loss over a 5 week storage frozen in Q-sepharose elution buffer at -20°C.

When comparing the two forms of r-MtL obtained from the fermentor broth isolated on Q-Sepharose, there are no significant differences seen in terms of SDS-PAGE, native 15 PAGE, native IEF, S-300 gel filtration, UV-visible spectrum, specific activity towards syringaldazine and ABTS, and deblocked N-terminus sequencing measurements. Likely, the different elution pattern on Q-Sepharose arises from some sort of differential glycosylation.

20

IV. USE OF MYCELIOPHTHORA LACCASE IN DYEING HAIR

The dyeing effect of *Myceliophthora* laccase is tested on various dye precursors and further on 0.1% p-phenylenediamine compared with a number of modifiers.

25 **Materials:**

Dye precursors:

0.1 % p-phenylene-diamine in 0.1 M K-phosphate buffer, pH=7.0)

0.1 % o-aminophenol in 0.1 M K-phosphate buffer, pH=7.0)

30

Enzymes:

Recombinant *Myceliophthora thermophila* laccase, 16 LACU/ml (in final dye solution).

Equipment:

Datacolor Textflash 2000 (CIE-Lab)

Assessment of the hair color

5 The quantitative color of the hair tresses is determined on a Datacolor Textflash 2000 by the use of CIE-Lab parameters L* ("0"=black and "100"=white) combined with a* ("--"=green and "+="=red).

10 **Results:**Dyeing effect

Tresses of blond European hair (1 gram) are used for testing *Myceliophthora thermophila* laccase in the context of oxidative hair dyeing. p-phenylene diamine and o-aminophenol are
15 used as the dye precursors.

Hair dyeing

4 ml dye precursor solution is mixed with 1 ml laccase on a Whirley mixer, applied to the hair tresses and kept at 30°C
20 for 60 minutes. The hair tresses are then rinsed with running water for about 3 minutes, pressed between two fingers, combed, and air dried.

The results of the dyeing effect test are displayed below in
25 Table 1 and 2.

Table 1

o-aminophenol	enzyme	L*	a*
Untreated blond hair	-	70.3	2.3
Laccase	+	57.7	15.3

30

Table 2

p-phenylenediamine	enzyme	L*	a*
Untreated blond hair	-	70.3	2.3
1.0 ml laccase	+	29.1	4.1

L*: 0=black, 100=white a*: --green, +red

5 Result of test:

From Table 1 and 2 it can be seen that the *Myceliophthora thermophila* laccase can be used for oxidative dyeing of hair.

10 Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern

15 Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 on May 25, 1994, and given the following accession number.

DepositAccession Number

E. coli JM101 containing

NRRL B-21261

20 pRaMB5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Novo Nordisk Biotech, Inc.
- (B) STREET: 1445 Drew Avenue
- (C) CITY: Davis, California
- (D) COUNTRY: United States of America
- (E) POSTAL CODE (ZIP): 95616-4880
- (F) TELEPHONE: (916) 757-8100
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(i) APPLICANT:

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- (C) CITY: Bagsværd
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- (E) POSTAL CODE (ZIP): DK-2880
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(ii) TITLE OF INVENTION: PURIFIED MYCELIOPHTHORA LACCASES AND NUCLEIC ACIDS ENCFODING SAME

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Novo Nordisk of North America, Inc.
- (B) STREET: 405 Lexington Avenue, Suite 6400
- (C) CITY and STATE: New York, New York
- (D) COUNTRY: U.S.A.
- (E) ZIP: 10174-6401

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: to be assigned
- (B) FILING DATE: 31-May-1995
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/253,781
- (B) FILING DATE: 03-June-1994

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Lowney, Karen A.
- (B) REGISTRATION NUMBER: 31,274
- (C) REFERENCE/DOCKET NUMBER: 4184.204-WO

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 212 867 0123
- (B) TELEFAX: 212 867 0298

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3187 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

ATC ACG GAG AGG GAG GAG GCA GCC GTG AAG GCT CGC CAG CAG AGC TGC Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln Gln Ser Cys 40 45 50	739
AAC ACC CCC AGC AAC CGG GCG TGC TGG ACT GAC GGA TAC GAC ATC AAC Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr Asp Ile Asn 55 60 65	787
ACC GAC TAC GAA GTG GAC AGC CCG GAC ACG GGT GTT GTT CGG CCG Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val Arg Pro 70 75 80	832
GTGAGTGCTC TCGTTAATTAA CGCTTCGGCG AGTTCGGCAG ATATATTAAA TACTGCACAC	892
CTAACCCAGGA GCTGACATGC GACAG TAC ACT CTG ACT CTC ACC GAA GTC GAC Tyr Thr Leu Thr Leu Thr Glu Val Asp 85 90	944
AAC TGG ACC GGA CCT GAT GGC GTC GTC AAG CAG AAG GTC ATG CTG GTT Asn Trp Thr Gly Pro Asp Gly Val Val Lys Glu Lys Val Met Leu Val 95 100 105	992
AAC GTACGGCACCC CTTTTCTTG TCCTAGGATC TGGGTGATGT GCGTCGTTGC Asn	1045
CCCTGAGAGA CTGACCGAGC CTTTGGCTGC AG AAT AGT ATA ATC GTAATTAATT Asn Ser Ile Ile 110	1099
ATACCGCCCT GCCTCCAGCA GCCCCAGCAG CTCGAGAAGG GTATCTGAAG TTAGTCAGGC	1159
CTGCTGACCT GACCGGGGCC AACCCATAG GGA CCA ACA ATC TTT GCG GAC TGG Gly Pro Thr Ile Phe Ala Asp Trp 115 120	1212
GCC GAC ACG ATC CAG GTA ACG GTC ATC AAC AAC CTC GAG ACC AAC GGC Gly Asp Thr Ile Gln Val Thr Val Ile Asn Asn Leu Glu Thr Asn Gly 125 130 135	1260
GTATGTCCTGC TGCTTGCTCT CTTGCTCTCC TCGTCCGCGA CTAATAATAA TATCAAATCG	1320
TGTGGAAAAAC AG ACG TCG ATC CAC TGG CAC GGA CTG CAC CAG AAG GGC Thr Ser Ile His Trp His Gly Leu His Gln Lys Gly 140 145	1368
ACC AAC CTG CAC GAC GGC GCC AAC GGT ATC ACC GAG TGC CCG ATC CCC Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu Cys Pro Ile Pro 150 155 160	1416
CCC AAG GGA GGG AGG AAG GTG TAC CGG TTC AAG GCT CAG CAG TAC GGG Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala Gln Gln Tyr Gly 165 170 175 180	1464
ACG AGC TGG TAC CAC TCG CAC TTC TCG GCC CAG TAC GGC AAC GGC GTG Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn Gly Val 185 190 195	1512
GTC GGG GCC ATT CAG ATC AAC GGA CCG GCC TCG CTG CCG TAC GAC ACC Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr Asp Thr 200 205 210	1560
GAC CTG GGT GTG TTC CCC ATC AGC GAC TAC TAC TAC AGC TCG GCC GAC Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Ser Ser Ala Asp 215 220 225	1608

GAG CTG GTG GAA CTC ACC AAG AAC TCG GGC GCG CCC TTC AGC GAC AAC Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala Pro Phe Ser Asp Asn 230 235 240 245	1656
GTC CTG TTC AAC GGC ACG GCC AAG CAC CCG GAG ACG GGC GAG GGC GAG Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu Thr Gly Glu Gly Glu 250 255 260	1704
TAC GCC AAC GTG ACG CTC ACC CCG GGC CGG CGG CAC CGC CTG CGC CTG Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg His Arg Leu Arg Leu 265 270 275	1752
ATC AAC ACG TCG GTC GAG AAC CAC TTC CAG GTC TCG CTC GTC AAC CAC Ile Asn Thr Ser Val Glu Asn His Phe Gln Val Ser Leu Val Asn His 280 285 290	1800
ACC ATG TGC ATC ATC GCC GGC GAC ATG GTG CCC GTC AAC GCC ATG ACG Thr Met Cys Ile Ile Ala Ala Asp Met Val Pro Val Asn Ala Met Thr 295 300 305	1848
GTC GAC ACC CTC TTC CTC GGC GTC GGC CAG CGT TAC GAT GTC GTC ATC Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg Tyr Asp Val Val Ile 310 315 320 325	1896
GAA GCC AAC CGA ACG CCC GGG AAC TAC TGG TTT AAC GTC ACA TTT GCC Glu Ala Asn Arg Thr Pro Gly Asn Tyr Trp Phe Asn Val Thr Phe Gly 330 335 340	1944
GGC GGC CTG CTC TGC GGC TCC AGG AAT CCC TAC CCG GCC GCC ATC Gly Gly Leu Leu Cys Gly Ser Arg Asn Pro Tyr Pro Ala Ala Ile 345 350 355	1992
TTC CAC TAC GCC GGC GCC CCC GGC CGC CCG CCC ACG GAC GAG GGC AAG Phe His Tyr Ala Gly Ala Pro Gly Pro Pro Thr Asp Glu Gly Lys 360 365 370	2040
GCC CCG GTC GAC CAC AAC TGC CTG GAC CTC CCC AAC CTC AAG CCC GTC Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro Asn Leu Lys Pro Val 375 380 385	2088
GTG GCC CGC GAC GTG CCC CTG AGC GGC TTC GCC AAG CGG GCC GAC AAC Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala Lys Arg Ala Asp Asn 390 395 400 405	2136
ACG CTC GAC GTC ACC CTC GAC ACC ACG GGC ACG CCC CTG TTC GTC TGG Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr Pro Leu Phe Val Trp 410 415 420	2184
AAG GTC AAC GCC AGC GCC ATC AAC ATC GAC TGG GGG AGG GCC GTC GTC Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp Gly Arg Ala Val Val 425 430 435	2232
GAC TAC GTC CTC ACG CAG AAC ACC AGC TTC CCA CCC GGG TAC AAC ATT Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro Pro Gly Tyr Asn Ile 440 445 450	2280
GTC GAG GTG AAC GGA GCT GAT CAG GTAAGAAAAA GGGGACCGCA GGGGTGCTGC Val Glu Val Asn Gly Ala Asp Gln 455 460	2334
TGCAAAGTACA CCTTGCTCGC CCTCCTGTTTC TTCCCTTAATA ACTACCTCCC AACCTCCCC CCTAAATTAAAT TCACTTTAAA GGCGGATCAA GACTGACCGA GCCCCCTCTC TTTGCAG	2394
TGG TCG TAC TGG TTG ATC GAG AAC GAT CCC GGC GCA CCT TTC ACC CTA Trp Ser Tyr Trp Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe Thr Leu 465 470 475	2451
	2499

CCG CAT CCG ATG CAC CTG CAC GTAAAGTTGGA TACATATATA TATATATATA Pro His Pro Met His Leu His 480	2550
TACATTGCTT TCCTGGCTCG CTCCCTTAAA TAAAATTAAA TAACCAAAAAA TAACAAAAAA AAG GGC CAC GAC TTT TAC GTG CTG GGC CGC TCG CCC GAC GAG TCG CCG Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp Glu Ser Pro 485 490 495	2610 2658
GCA TCC AAC GAG CGG CAC GTG TTC GAT CCG GCG CCG GAC GCG GGC CTG Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp Ala Gly Leu 500 505 510 515	2706
CTG AGC GGG GCC AAC CCT GTG CGG CGG GAC GTG TCG ATG CTG CCG GCG Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met Leu Pro Ala 520 525 530	2754
TTC GGG TGG GTG GTG CTG TCC TTC CGG GCC GAC AAC CCG GGC GCC TGG Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro Gly Ala Trp 535 540 545	2802
CTG TTC CAC TGC CAC ATC GCC TGG CAC GTC TCG GGC GGC CTG GGC GTC Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly Leu Gly Val 550 555 560	2850
GTC TAC CTC GAG CGC GCC GAC GAC CTG CGC GGG GCC GTC TCG GAC GCC Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val Ser Asp Ala 565 570 575	2898
GAC GCC GAC CTC QAC CGC CTC TGC GCC GAC TGG CGC CGC TAC TGG Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg Arg Tyr Trp 580 585 590	2946
CCT ACC AAC CCC TAC CCC AAG TCC GAC TCG GGC CTC AAA CAC CGC TGG Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys His Arg Trp 595 600 605	2994
GTC GAG GAG GGC GAG TGG CTG GTC AAG GCG TGACCGAAGG AGGAAAAAGG Val Glu Glu Gly Glu Trp Leu Val Lys Ala 610 615	3044
AAACAAAGAG GGGGGGGGGG GCTAGTTCCCT ATTTTTGCTT TTTTTTTTTG TTCTTGCTCT TGTGCTGGCG GTTCCCTGGT AAAGGAGAAG GGGGCCCAA GTTCGAGTGG GTGTGTGATC GGGTAAATAT TATCAAGAGA TCT	3104 3164
	3187

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 620 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Myceliophthora thermophila
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Ser Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr
1 5 10 15

Pro Ser Val Ala Ala Ala Pro Pro Ser Thr Pro Glu Gln Arg Asp Leu
 20 25 30
 Leu Val Pro Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln
 35 40 45
 Gln Ser Cys Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr
 50 55 60
 Asp Ile Asn Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val
 65 70 75 80
 Arg Pro Tyr Thr Leu Thr Leu Thr Glu Val Asp Asn Trp Thr Gly Pro
 85 90 95
 Asp Gly Val Val Lys Glu Lys Val Met Leu Val Asn Asn Ser Ile Ile
 100 105 110
 Gly Pro Thr Ile Phe Ala Asp Trp Gly Asp Thr Ile Gln Val Thr Val
 115 120 125
 Ile Asn Asn Leu Glu Thr Asn Gly Thr Ser Ile His Trp His Gly Leu
 130 135 140
 His Gln Lys Gly Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu
 145 150 155 160
 Cys Pro Ile Pro Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala
 165 170 175
 Gln Gln Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr
 180 185 190
 Gly Asn Gly Val Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu
 195 200 205
 Pro Tyr Asp Thr Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Tyr
 210 215 220
 Ser Ser Ala Asp Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala Pro
 225 230 235 240
 Phe Ser Asp Asn Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu Thr
 245 250 255
 Gly Glu Gly Glu Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg His
 260 265 270
 Arg Leu Arg Leu Ile Asn Thr Ser Val Glu Asn His Phe Gln Val Ser
 275 280 285
 Leu Val Asn His Thr Met Cys Ile Ile Ala Ala Asp Met Val Pro Val
 290 295 300
 Asn Ala Met Thr Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg Tyr
 305 310 315 320
 Asp Val Val Ile Glu Ala Asn Arg Thr Pro Gly Asn Tyr Trp Phe Asn
 325 330 335
 Val Thr Phe Gly Gly Leu Leu Cys Gly Gly Ser Arg Asn Pro Tyr
 340 345 350
 Pro Ala Ala Ile Phe His Tyr Ala Gly Ala Pro Gly Gly Pro Pro Thr
 355 360 365

Asp Glu Gly Lys Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro Asn
370 375 380

Leu Lys Pro Val Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala Lys
385 390 395 400

Arg Ala Asp Asn Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr Pro
405 410 415

Leu Phe Val Trp Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp Gly
420 425 430

Arg Ala Val Val Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro Pro
435 440 445

Gly Tyr Asn Ile Val Glu Val Asn Gly Ala Asp Gln Trp Ser Tyr Trp
450 455 460

Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe Thr Leu Pro His Pro Met
465 470 475 480

His Leu His Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp Glu
485 490 495

Ser Pro Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp Ala
500 505 510

Gly Leu Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met Leu
515 520 525

Pro Ala Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro Gly
530 535 540

Ala Trp Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly Leu
545 550 555 560

Gly Val Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val Ser
565 570 575

Asp Ala Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg Arg
580 585 590

Tyr Trp Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys His
595 600 605

Arg Trp Val Glu Glu Gly Glu Trp Leu Val Lys Ala
610 615 620

What we claim is:

1. A DNA construct containing a sequence encoding a *Myceliophthora laccase*.

5

2. The construct of Claim 1 which comprises a sequence encoding a *Myceliophthora thermophila laccase*.

10 3. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.

15

5. The construct of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21261.

6. A substantially pure *Myceliophthora laccase* enzyme.

20

7. The enzyme of Claim 6 which is a *Myceliophthora thermophila laccase*.

25

8. The enzyme of Claim 6 which comprises the amino acid sequence depicted in SEQ ID NO. 2, or a sequence with at least about 80% homology thereto.

9. A recombinant vector comprising an DNA construct containing a sequence encoding a *Myceliophthora laccase*.

30

10. The vector of Claim 9 in which the construct is operably linked to a promoter sequence.

11. The vector of Claim 10 in which the promoter is a fungal or yeast promoter.

12. The vector of Claim 11 in which the promoter is the 5 TAKA amylase promoter of *Aspergillus oryzae*.

13. The vector of Claim 11 in which the promoter is the glucoamylase (gluA) promoter of *Aspergillus niger* or *Aspergillus awamori*.

10

14. The vector of Claim 9 which also comprises a selectable marker.

15. The vector of Claim 14 in which the selectable marker 15 is selected from the group consisting of *amdS*, *pyrG*, *argB*, *niaD*, *sc*, and *hygB*.

16. The vector of Claim 14 in which the selectable marker 20 is the *amdS* marker of *Aspergillus nidulans* or *Aspergillus oryzae*, or the *pyrG* marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.

17. The vector of Claim 14 which comprises both the TAKA 25 amylase promoter of *Aspergillus oryzae* and the *amdS* or *pyrG* marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

18. A recombinant host cell comprising a heterologous 30 nucleic acid construct containing a nucleic acid sequence encoding a *Myceliophthora laccase*.

19. The cell of Claim 18 which is a fungal cell.

20. The cell of Claim 19 which is an *Aspergillus* cell.

- 21. The cell of Claim 18 in which the construct is integrated into the host cell genome.
- 5 22. The cell of Claim 18 in which the construct is contained on a vector.
- 23. The cell of Claim 18 which comprises a construct containing a sequence encoding the amino acid sequence
10 depicted in SEQ ID NO. 2..
- 24. A method for obtaining a laccase enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Myceliophthora* 15 laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.
- 25. A *Myceliophthora* enzyme obtained by the method of Claim 24.
20
- 26. An ascomycete or deuteromycete laccase having a specific activity of at least 30 SOU/mg on syringaldazine at optimum pH.
- 25 27. A method of enhancing yield of active recombinant copper-containing enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a sequence encoding a copper containing enzyme, under conditions conducive to expression of the enzyme, in the 30 presence of at least about 0.02mM copper.
- 28. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Myceliophthora* laccase.

29. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Myceliophthora* laccase.

5

30. A method for oxidizing dyes or dye precursors which comprises contacting the dye with a *Myceliophthora* laccase.

31. A method for dyeing hair which comprises contacting a
10 *Myceliophthora* laccase, in the presence or absence of at least one modifier, with at least one dye precursor, for a time and under conditions sufficient to permit oxidation of the dye precursor to a dye.

15 32. The method of claim 31 in which the dye precursor is selected from the group consisting of a diamine, aminophenol, and a phenol.

33. The method of claim 31, wherein the modifier, when
20 used, is a meta-diamine, a meta-aminophenol or a polyphenol.

34. The method of claim 32 in which the dye precursor is a primary intermediate selected from the group consisting of an ortho- or para-diamine or aminophenol.

25

35. The method of claim 31 in which more than one dye precursor is used.

36. The method of claim 31 in which more than one modifier
30 is used.

37. The method of claim 31 in which both a primary intermediate and a modifier are used.

38. A dye composition comprising a *Myceliophthora laccase* combined with at least one dye precursor.

39. A dye composition comprising a *Myceliophthora laccase* combined with at least one primary intermediate and at least one modifier.
5

40. A container containing a dye composition comprising a *Myceliophthora laccase* and at least one dye precursor in an
10 oxygen-free atmosphere.

41. The container of claim 40 which contains at least one primary intermediate dye precursor combined with at least one modifier.
15

42. A method of polymerizing or oxidizing a phenolic or aniline compound which comprises contacting the phenolic or aniline compound with a *Myceliophthora laccase*.

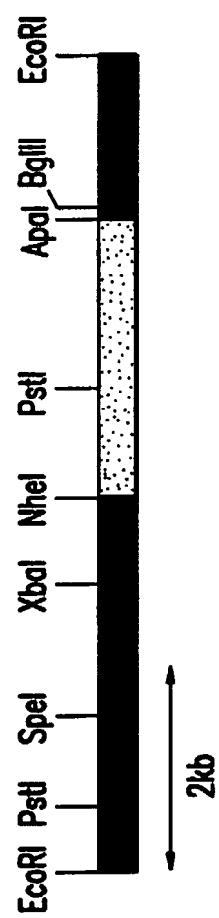


FIG. 1

1/5

SUBSTITUTE SHEET (RULE 26)

gctagcttcgttggcacccgtcglttcgcccccccccccccctccctcaacccctgagtagtcggctaaaggcatccctc 80
atctggcttgtgaggtaacgtccctccagcagatgacagtatcgagcgatgtatccaccacccagaagggggggggg 160
 gatgcgcgcatgcctccaacatccctggcgcttagagacgcgcgcattccatcacacccgagcacglcc 240
 cggaccggctcccttccaccccccgcgtccctccggaggatgtgatcacgatatttcggatgtggaaaggggagagaaagg 320
 agggggggggggcgaaaactgttgatocgagctgcgccttttccaaacatcgagaaacaggaaatgtcgatgtggatcg 400
cgtaaatgtctataaaacgaggctcccttcgtcgacttgtctcaggatctctctcgccacaccaaggccatctt 480
 gcctgagccacctgagccacccctcaactcatcatcttcagtcagtcgttcaatgtgatcttcgtatctatcgag 560
tccgcitcccgcccttaccacaaacATGAAGTCCTTCATCAGCGCCGCGACGCCCTTGGTGGCATTCACCCCTAGC 640
 MetLysSerPhenLeSerAlaAlaThrLeuLeuValGlyIleLeuThrProSer -30
 GTTGCTGCTGCCCTCCATCCACCCCTGAGCACGCCGACCTGCTCGTCCCCATCACGGAGGGAGGCAGCCGTGAA 720
 ValAlaAlaAlaProProSerThrProGluGlnArgAspLeuLeuValIProleThrGluArgGluGluAlaAlaValIy 73
 GGCTCCCAAGCAGCTGCAACACCCCCCAGCAACCGGGCTGCTGGACTGACGGATAACGACATCAACACCGACTACGAAG 800
 sAlaArgGlnGlnSerCysAsnThrProSerAsnArgAlaCysTrpThrAspGlyTyrAspIleAsnThrAspTyrGluV 25
 TGGACAGCCCCGACACGGGTGTTGGCCGgtgatgtctcgatataatcgcttcggcgatgtgcgcagatata 880
 aIAspSerProAspThrGlyValValArgPro 35
aatactgcacccatcgaggatgtacatgcgcacagTACACTCTGACTCTACCGAACGACTGGACCCGACCTG 960
 TyrThrLeuThrGluValAspAsnTrpThrGlyProA 50
 ATGGCGTCGTCAAGGAGAACGTCATGCTGGTTAACAGtacggcaccccttttcgttccatgtggatctggatgtgcgt 1040
 spGlyValValLysGluLysValMetLeuValAsnA 62
cgttgcctcgaggatgtacccgccttggctgcacatAGTATAATCGttaatataatccgcctgcctccac 1120
 snSerIleIleG 66
 cagccccagcagctcgagaaagggtatcgaaatgtacggcctgcgtacccgcctggccaaacccaccatagGACCAA 1200
 lyProt 68
 CAATCTTGGGACTGGGGCACACGATCCAGTAACGGTCATCAACAACCTCGAGACCAACCGgtatgtctgtgttg 1280
 hrIlePheAlaAspTrpGlyAspThrIleGlnValThrValIleAsnAsnLeuGluThrAsnG 89

FIG.2A

cctctttgtctcccgactaaataatcaactcgltggaaaaacogCACGTCGATCCACTGGCACCGACT yThrSerIleHisTrpHisGlyLe	1360 97
GCACCAAGGGCACCAACCTGCACCGACGGCGCCAACGGTATCACCGAGTGGCGATCCCCCCCAGGGAGGAAGG uHisGlnLysGlyThrAsnLeuHisAspGlyAlaAsnGlyIleThrGluCysProIleProProLysGlyGlyArgLysV	1440 124
TGTACCGGTTCAAGGCTCAGCAGTACGGGACGAGCTGGTACCACTCGCACTTCTCGGCCAGTACGGCAACGGCGTGGTC aITyrArgPheLysAlaGlnGlnTyrGlyThrSerTrpTyrHisSerHisPheSerAlaGlnTyrGlyAsnGlyValVal	1520 150
GGGGCCATTCAAGATCAACGGACGGGCTCGCTGCCGTACGACACCGACCTGGGTGTGTTCCCCATCAGCGACTACTACTA GlyAlaIleGlnIleAsnGlyProAlaSerLeuProTyrAspThrAspLeuGlyValPheProIleSerAspTyrTyrTy	1600 177
CACGCTGGCGACGGCTGGTGGAACTCACCAAGAACCTGGCGGCCCTTCAGCGACAACGTCCTGTTAACCGCACGG rSerSerAlaAspGluLeuValGluLeuThrLysAsnSerGlyAlaProPheSerAspAsnValLeuPheAsnGlyThrA	1680 204
CCAAGCACCCGGAGACGGCGAGGGCGACTACGCCAACGCTGACGCTCACCCCGGGCCGGCGCACCGCCTGCGCTGATC IaLysHisProGluThrGlyGluGlyGluTyrAlaAsnValThrLeuThrproGlyArgArgHisArgLeuArgLeuIle	1760 230
AACACGTCGGTCCAGAACCACTTCCAGCTCGCTCGCAACCACACCATGTGCATCATGCCCGCCGACATGGTCCCCGT AsnThrSerValGluAsnHisPheGlnValSerLeuValAsnHisThrMetCysIleIleAlaAlaAspMetValProVa	1840 257
CAACGCCATGACGGTCGACAGCCTTCCCTGGCGTGGCCAGCGTTACGATGTCGTATCGAAGCCAACCGAACGCCCG IAasnAlaMetThrValAspSerLeuPheLeuGlyValGlyGlnArgTyrAspValValIleGluAlaAsnArgThrProG	1920 284
GGAACTACTGGTTAACCTCACATTGGCGCGGCTGCTCGCGCGCTCCAGGAATCCCTACCCGGCCGCCATCTC IyAsnTyrTrpPheAsnValThrPheGlyGlyLeuLeuCysGlyGlySerArgAsnProTyrProAlaAlaIlePhe	2000 310
CACTACGGCGGGCGCCCCCGGCGGCCACGGACGGAGGGCAAGGCCCCGGTCGACCAACTGCCCTGGACCTCCCCAA HisTyrAlaGlyAlaProGlyGlyProProThrAspGluGlyLysAlaProValAspHisAsnCysLeuAspLeuProAs	2080 337
CCTCAAGCCCGTGTGGCCCGCGACGTGCCCTGAGCGCTCGCCAAGGGCGACAAACGCTCGACGTCAACCTCG nLeuLysProValValAlaArgAspValProLeuSerGlyPheAlaLysArgAlaAspAsnThrLeuAspValThrLeuA	2160 364
ACACCAACGGGCACGCCCTGTCGCTGGAAAGTCACGGCAGGCCATCAACATCGACTGGGGAGGGCCGCTCTCGAC spThrThrGlyThrProLeuPheValTrpLysValAsnGlySerAlaIleAsnIleAspTrpGlyArgAlaValValAsp	2240 390
TACCTCCTCACGGAGAACACCAGCTCCACCCGGTACAACATTGTCAGGTGAACGGAGCTGATCAGgtaaaaaaaag TyrValLeuThrGlnAsnThrSerPheProProGlyTyrAsnIleValGluValAsnGlyAlaAspGln	2320 413
gggaccgcagggtgctgctgcoagtacaccctgctgccclctgtlcllccttaataactacctccaaacctcccc cacttaattcaclltaaggccgtcaagactgaccgagccccctcttgcagTGCTCTACTGGTTGATCGAGAAC	2400 2480
TrpSerTyrTrpLeuIleGluAsn	421

FIG.2B
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FIG.2C

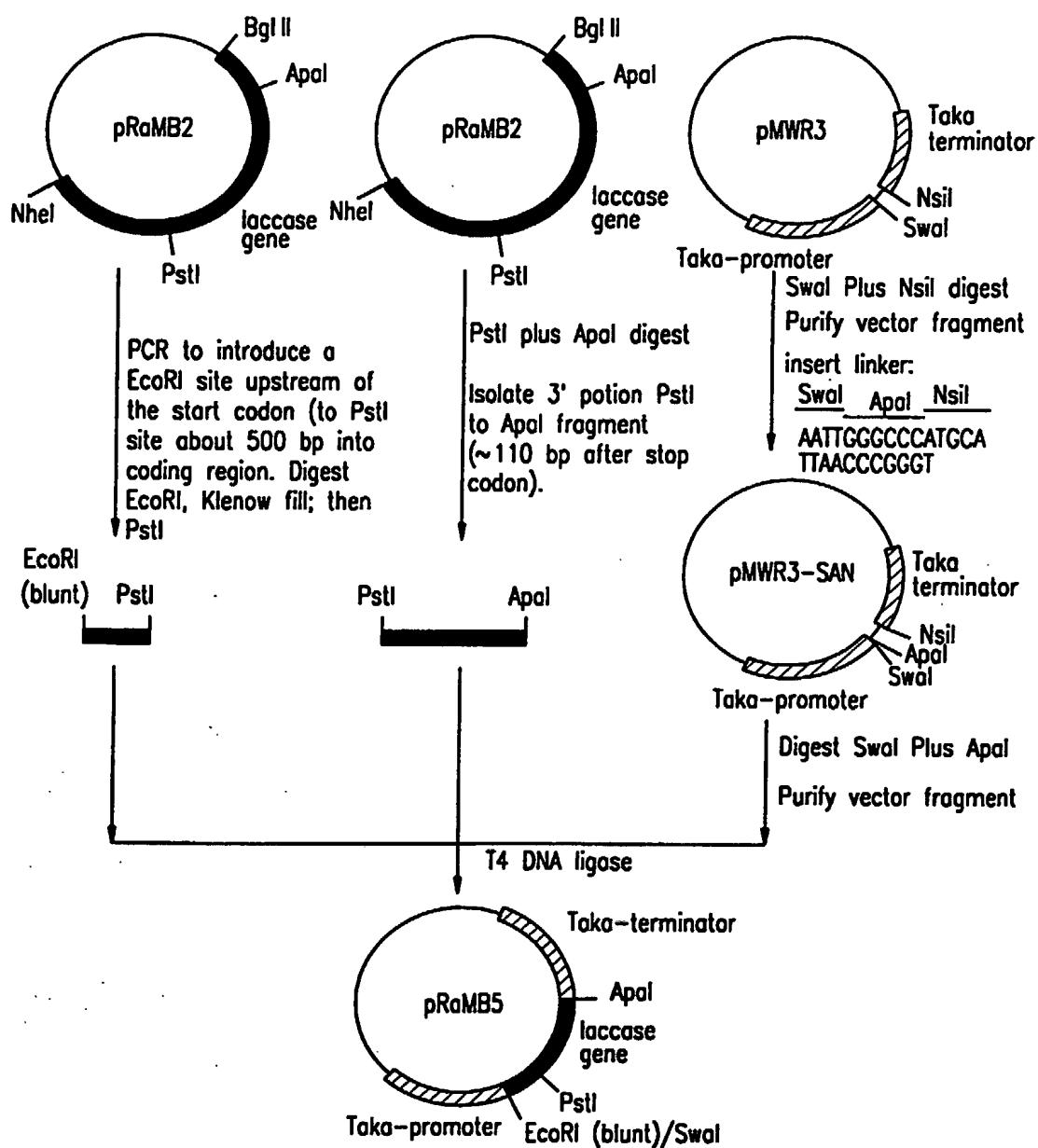


FIG.3

INTERNATIONAL SEARCH REPORT

Internat'l Application No PCT/US 95/06815
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A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/53 C12N1/15 A61K7/13 A61K7/06 D21C5/00
 //((C12N1/15, C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N A61K D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, O, X	ABSTRACTS OF PAPERS, vol.209, no.1-2, April 1995, ANAHEIM, CA BERKA R. ET AL. 'Cloning of laccases from the thermophilic fungi Myceliophthora thermophila and Scytalidium thermophilum and their heterologous expression in <i>Aspergillus oryzae</i> ' see BIOT 196 ---	1, 2, 6, 7, 9, 10, 18-20, 24-26
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.263, no.2, 1988, BALTIMORE, MD US pages 885 - 896 GERMANN U. ET AL. 'Characterization of two allelic forms of <i>Neurospora crassa</i> laccase' see page 885, right column --- -/-	26

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INTERNATIONAL SEARCH REPORT

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C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 317 243 (KYOWA MEDEX CO. LTD.) 24 May 1989 ---	
P,X	WO,A,95 01426 (NOVO NORDISK A/S) 12 January 1995 see page 19; claim 22 -----	6,7,25, 26

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/06815

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0317243	24-05-89	JP-A-	1128797	22-05-89
		DE-D-	3851304	06-10-94
		DE-T-	3851304	26-01-95
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WO-A-9501426	12-01-95	AU-B-	6924594	24-01-95

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